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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 :
C12N 15/00, A61K 35/52
A01K 1/00

A1

(11) International Publication Number: WO 87/ 05325

(43) International Publication Date: 11 September 1987 (11.09.87)

(21) International Application Number: PCT/EP87/00123

(22) International Filing Date: 2 March 1987 (02.03.87)

(31) Priority Application Numbers: P 36 06 891.8
P 36 36 991.8

(32) Priority Dates: 3 March 1986 (03.03.86)
30 October 1986 (30.10.86)

(33) Priority Country: DE

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(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.

Published
*With international search report.*
*Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.*

(54) Title: A METHOD FOR TRANSFERRING ORGANIC AND/OR INORGANIC SUBSTANCES TO EGG CELLS AND/OR SOMATIC CELLS OF ANIMALS AND COMPOSITIONS FOR USE THEREIN

(57) Abstract

A method of transferring organic and/or inorganic substances to egg cells and/or somatic cells of animals by combining sperms of the respective type, optionally modified by chemical or physical means, with vesicles or granulae containing the desired organic or inorganic substances and subsequently contacting the loaded sperms with egg cells or somatic cells under intracorporal or extracorporal conditions. The invention also relates to compositions suitable for use in the method and to precursors of such compositions. Finally, the invention relates to transgenic animals produced by the method.

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# A METHOD FOR TRANSFERRING ORGANIC AND/OR INORGANIC SUBSTANCES TO EGG CELLS AND/OR SOMATIC CELLS OF ANIMALS AND COMPOSITIONS FOR USE THEREIN

The invention relates to a method of transferring organic and/or inorganic substances to egg cells and/or somatic cells of animals, compositions suited for use in the method, precursors of such compositions, and transgenic animals obtained by the method.

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Disclosed herein is a method of transferring organic and/or inorganic substances by means of sperms to the respective target cells. The substances to be transferred are protected from dilution, enzymatic or other modification by being entrapped in vesicles or granulae. The envelopes are then bound to sperms. The sperms carry the vesicles or granulae to the target cell, preferably an egg cell, penetrating the outer membranes thereof. Vesicle contents or granula components are released within the cell where they exert their specific effects. The method is particularly suited for transferring nucleic acids into egg cells so as to produce transgenic organisms as well as for studies of teratology and gene regulation in the early stages of embryogenesis.

Up to now, gene transfer to an organism has been accomplished exclusively by manipulation of ovum or embryo or with the aid of viruses (Palmiter et al., 1982: Nature, 300:611; Brackett et al., 1971: PNAS 68 (2):353). Use of complete viruses as gene vectors is extremely problematic and should be firmly rejected in the interest of experimentator, animal breeder, consumer and, last but not least, the test animal itself.

Methods of producing transgenic animals have been summarized systematically by Palmiter and Brinster (1986,Ann. Rev. Genet. 20). Most commonly employed is pronucleus injection as illustrated in Figure 1.

(1) The most widely accepted technique so far is microinjection of genes (dissolved in physiological buffer) into one of the pronuclei of the fertilized egg. Several thousands of transgenic mice (as the classical test animal) as well as a small number of transgenic sheep, pigs and rabbits have been produced in this manner since 1980.

The injection procedure does however require highly sophisticated instruments and extreme skill. Factors influencing integration of the injected gene are: DNA concentration, type of buffer solution, injected cell, form of plasmid , and origin of the embryo (strain of mice). Length of the DNA molecule (0.5 to 10.0 kb), on the other hand, is without influence on integration frequency.

The integration mechanism of microinjected DNA is not known. Tandem configuration in the presence of several copies is observed in most cases, but various loci on a number of chromosomes are known, too. As integration rate of linear DNA is five times higher than that of circular DNA, the ends of the fragments are apparently involved in integration.

As a rule, integrated extraneous DNA remains stable for many generations; amplification or deletion is noted occasionally.

According to a further method of producing transgenic animals, DNA of the desired type was injected into the cytoplasm of the zygote, the blastomeres of the bicellular organism, or the blastocoele of the blastocyst. This method has less chance of success, and formation of mosaics is very likely.

(2) A very inefficient method of producing transgenic animals is nucleus replacement. Here, the nuclei of totipotent cells transfected in vitro must be isolated by micromanipulation, the plasma membrane being removed together with the major portion of the cytoplasm. The isolated nucleus is then transferred with a micropipette to a freshly fertilized egg. The nucleus is deposited, and female as well as male pronucleus

are removed by suction when the pipette is withdrawn from the egg. The egg cell now has a diploid nucleus containing a (previously in vitro transfected) gene. Extreme skill in the techniques of micromanipulation is required for this method, too, which, though being costly, provides little chances of success.

(3) In another method, teratocarcinoma cells are transfected and subsequently transferred as gene vehicles into embryos (blastocysts). Chimeras with one transgenic cell line are obtained in this manner. The problems regarding selectivity of expression and diploidy of the desired cells are, however, substantial, and transmission into the germline is a mere coincidence.

(4) The genetically manipulated retrovirus used in a further transfer method is capable of infecting embryos. As potential expression of retroviral native genes cannot yet be eliminated reliably, the risk involved in manipulating viruses precludes their use in practice.

As regards the problems of controlled selective integration, it could be proved in experiments on cultured cells that insertion of extraneous hereditary material at some accurately defined point of a complete genome is possible (Smithies et al., 1985, Nature 317: 230). Depending on the structure of the plasmide or vector used, a new gene may be introduced, an already present gene may be destroyed (the object being elimination of a negative mutation), or a gene may be replaced in this manner.

All of the discussed methods of producing transgenic animals require eggs or embryos in vitro, meaning that donor animals must be stimulated hormonally to produce a maximum number of eggs and ovulate at a certain time. Hormonal stimulation is in itself an artificial intervention affecting the hormonal balance of the animal and resulting in formation of antibodies against the respective hormone (PMSG = pregnant mare serum gonadotropin);

it also is a costly procedure. Moreover, the animals thus treated must be closely watched by veterinarian and attending staff over a prolonged period of time prior to oestrus.

As a rule, the artificially inseminated animals must undergo surgery so that the embryos may be recovered by flushing. Cows are the only animals where this may be done without an operation but the person conducting flushing needs special training. A catheter is placed near the end of the uterus and sealed against the cervix with a rubber bulb. Buffer solution is then pumped into the uterus, and the embryos will be washed out when this solution is removed again by suction; the embryos are so tiny that they can only be detected with a microscope.

The embryos of pigs and sheep are washed out in a similar manner from the surgically exposed uterus. However, as the peritoneum of the donor animal must be aseptically opened from the abdominal side, elaborate and costly anesthetic techniques, an operating table, expensive surgical instruments and a well trained operating team are required.

When, after time-consuming search with the microscope, the embryos are finally available, actual gene transfer according to any one of the above discussed methods may start at last.

About 20 to 80 % of the embryos subjected to prior gene transfer methods are not even suited for retransfer into a foster mother as their plasma or nucleus membranes have been destroyed by the injection.

The remaining uninjured embryos may now be retransferred into a foster mother. With cows, this may again be accomplished by non-surgical means but the receiver animal must be hormonally synchronized by elaborate preparatory treatment to adjust the physiological conditions in its uterus to those of a pregnant animal. For pigs and sheep, an operation is again required (costly and time-consuming) for transferring the embryos to the animal

that is to carry them to the full term. The young finally born are but a small percentage of the originally available embryos. Of these young ones, a maximum of 25 % (mice) and less than 0.1 to 1 % of sheep, pigs and rabbits are actually transgenic.

The method of the invention now serves for incorporating specific substances into animal cells, the object being a modification of the respective cells or investigation of the effects exerted within the cells by certain substances.

Cells that are of greatest economic and scientific value for such applications and investigations are e.g. the hardly accessible egg cells. Methods involving egg cells are therefore specific embodiments of the invention,

The method of the invention is based on the long-approved biological principle of DNA transfer, namely use of sperms as transfer vehicles. It also uses the firmly established routine of artificial insemination (particularly of domestic animals), a detailed discussion of which is not necessary here.

Making use of natural fertilization phenomena and artifical insemination techniques, sperms are advantageously incubated for a short period of time together with synthetically prepared vesicles or granulae which are adsorbed on the sperms under the chosen conditions. The vesicle-loaded sperms are subsequently deposited at the fertilization site where they may enter into the egg cells. The vesicles are prepared in the presence of nucleic acids coding for one or more genes to be transferred, or in the presence of other substances that are to be transferred into egg cells or somatic cells. On formation of the vesicles or granulae, the substances to be transferred become entrapped therein, thus being protected during transport to the egg cells. Sperms then take the vesicles into the cytoplasm of the target cells. Here, the granulae dissolve, or the vesicles undergo lysis with cell-inherent enzymes, releasing the entrapped substances. When nucleic acids are transferred in this manner and integrated in the genome of the zygote, the new gene(s) may be present in all cells of the developing

organism. Once established in the germinal cell, the genes thus transferred will be transmitted to all descendants of the respective organism.

A central aspect of the invention is the following: Sperms transfer substances bound to their surface, e.g. into egg cells. To make transportation by sperms possible, the respective substance must be attached to the sperm surface. It must also be entrapped so as to prevent dilution or modification through environmental factors during transport. The active substances are therefore provided with a suitable envelope, the envelope being then attached to the sperm surface. Materials used for this purpose must be capable of firmly connecting sperm surface and envelope. On contact with a target cell, the sperm enters the cell, releasing the transferred substances within the cell interior. As shown hereinafter, the envelopes for the substances to be transferred may always be prepared in accordance with analogous techniques.

The sperms (= spermatozoa) of all higher multicellular organisms differ in form as well as in molecular composition. Simply expressed, sperms are composed of a head and a tail portion. The head portion contains hereditary material in condensed form while the tail portion constitutes a substantially self-sufficient locomotory organ. Sperms are covered by a highly differentiated plasma membrane (plasmalemma) extending over the entire sperm structure (head and tail portion). Physico-chemical characteristics of the plasma membrane vary in accordance with the maturity of the sperms (epididymal or ejaculated). A loss in specific sperm-covering molecules as well as an increase in the concentration of new substances on the spermal surface during spermiogenesis has been proved. This is particularly evident from the steady increase in concanavalin A receptors on the surface of the sperms during maturation in the epididymis (Fournier-Delpech et al., 1977, Ann.Biol.Anim.Biochim.Biophys. 17:207).

Studies of the glycoproteins on the surface of epididymal sperms of rats have shown that a certain type of glycoprotein is present

only in the plasma membrane of the tail portion. This and several other types of sperm-covering glycoproteins secreted in the male genital tract are similar to fibronectins, a large group of adhesive glycoproteins found on cell surfaces, in connective tissue and some body fluids. All of these glycoproteins are associated with membranes and play a part in cellular adhesion.

More detailed information on the biochemical composition of spermal plasma membranes is mostly based on indirect evidence such as differences in charge and orientation in an electric field, agglutination behavior, local expression of H-Y antigen, change in behavior after treatment with neuraminidase, binding capacity for colloidal iron and, above all, different binding affinities of the various regions of the plasma membrane for lectins that bind to carbohydrate side-chains. With sperms, too, such carbohydrate side chains serve a function in cell recognition. Enzymes producing carbohydrate side chains are primarily glycosyltransferases and glucosidases characterized by high cell-specific activity. This is the reason for the remarkable differences in chemical structure and function of carbohydrate groups in individual types of cells. Some of these enzymes may even be actively absorbed by cells from the extracellular environment. In view of the various accessory secretions contacting the sperms within the reproductive tract, this fact is of particular importance.

Regional differentiation of the plasma membrane is manifest, too, in the very characteristic distribution of various membrane-bound enzymes such as ATPase and other phosphatases. A number of different substances (certain toxins, alkaloids, ionophores) show a tendency of binding precisely to that ATPase on the sperm membrane. Similar receptor mechanisms account for the good binding of a plurality of substances such as thalidomide, tetracycline and alpha-bungarotoxin to the spermal surfaces.

Spermal membranes are also capable of binding a number of steroid hormones and so-called "second messengers" such as cyclic AMP. Bovine sperms absorb a variety of steroids, of which

progesterone forms the strongest bond, followed by oestradiol, dihydrotestosterone, androstanediol and eticholanone.

A further component of the outer plasma membrane of sperms is the so-called membrane-bound, substrate-binding transport protein (carboglutelin). This protein binds and absorbs a variety of sugars from the liquid surrounding the sperms, thus supplying the sperms with the required energy.

Regional binding characteristics of the spermal plasma membrane have also been demonstrated by other methods. The bindings sites for prostaglandin and lactate-dehydrogenase on sperms of rabbits and mice were localized by immunofluorescence. Diiodofluorescein-isothiocyanate and radio-iodination gave an understanding of regional differentiation of the plasma membrane and the characteristics of surface proteins and lipids in the sperms of sea-urchin, golden hamster and bull. Fluorescent chloro-tetracycline was successfully used with sperms of mice to show changes in the plasma membrane during acrosome reaction and fertilization. Valueable findings were made in experiments with 1-anilinonaphthalene-8-sulphonate, especially with respect to substances from the environment that are deposited on the sperm surface (Edelmann and Millette, 1971, PNAS 68:2436; Mercado and Rosado, 1973, Biol. Reprod. 14:632).

As shown above, there exist numerous possibilities of attaching extraneous substances to the surface of sperms. Of decisive importance is the molecular surface composition of the particular sperms used, this composition depending in turn on the maturation stage of the sperms and the species from which the sperms were taken. In some cases, modification of the surfaces of the spermal plasma membranes prior to loading with envelopes may be advantageous. Enzymatic treatment may either remove structural surface elements that interfere with binding, or synthesize additional molecules on the surface to improve binding capacity.

In some cases (gene substitution, somatic gene therapy) where substances are to be transferred by sperms to somatic cells, it

might be advisable to first eliminate the haploid genome of the sperms by ionizing radiation so as not to introduce additional genetic material into the diploid target cell. Use of sperms as "genome-free" transfer vehicles is possible, especially if the sperms have first been subjected to high-intensity radiation of short wavelength.

The variety of possible bindings between sperm membrane and entrapped substances contemplated according to the invention will be illustrated below.

The surfaces of biological structures such as the outer plasma membranes of sperms, show external three-dimensional structural elements (groups of molecules) that protrude from the surface (membrane matrix). The membrane as such (basic structure) is composed of a lipid double layer, both surfaces of that layer being hydrophilic. Anchored to this lipid double layer are, among other components, various types of proteins through their lipophilic amino acid sequences. Hydrophilic side chains of proteins and lipids protrude from the surface; they usually are oligo- or polysaccarides or hydrophilic amino acid sections of proteins. These elements are of a special three-dimensional structure and further characterized by electric charges, hydrophilic or hydrophobic interaction, van der Waals' forces, and chemico-physical parameters of the environment. Many of these "protruding" elements are suited as binding points for substances approaching the plasma membrane from the outside. Similar spatial structural elements are found on the surface of the casings used in the method of the present invention.

The method of the invention is based on the concept that certain protruding three-dimensional structural elements on the surfaces of sperms might be bound to structural elements on the surface of the envelopes. Depending on nature and character of the spermal plasma membrane, type of envelope and type of binding, connection with the plasma membrane of the sperms may result in addition (adsorption) or, when the two units merge, in fusion and resulting endocytosis of the envelope contents. Sperms constitute the substantially

"invariable" element in the method of the invention as they vary only in accordance with their state of maturation and the species from which they were obtained; in other words: depending on the species, sperms show a more or less differently composed surface structure. Thus, depending on the species, other forms of binding become possible even though a large number of sperm types (higher vertebrates) show some common surface structures such as certain terminal sugar groups. In principle, it may be said that the specific surface characteristics of the sperms used for the respective purpose imply the composition of the envelope (with respect to surface characteristics) and, thus, the best suited form of binding between envelopes and sperms. But there also exists the possibility of intentionally altering surface characteristics of the sperms (modification). For instance, the large number of terminal sialinic acid groups on the sperm surface may be cleaved by incubating the sperms with the enzyme neuraminidase. In this manner, negative surface charge will be reduced and many, often distinctive sugar molecules previously hidden under sialinic acid groups (cryptantigens) are now exposed. These terminal sugar molecules again permit new forms of binding. Enzymatic treatment may also produce a "new coating" on the sperm surface. Glycosyltransferases, for instance, transfer specific sugar molecules to the surface of the sperms, thus offering other binding possibilities.

The forms of binding suited for the method of the invention may be divided in two groups:

(a) Direct binding of envelopes to sperms.

A structural element on the surface of an envelope ("lock") corresponds sterically (as a structure in space) as well as physicochemically (e.g. opposite charge) to an elevated structural element ("key") on the sperm membrane (key-lock principle). The more precisely the key fits the lock, the firmer the bond will be. Phenomena of a purely physical nature such as oppositely charged sperm surfaces and envelopes, also result in direct binding. As an example of direct binding, specific virus receptors may be mentioned that bind to precisely definable structural elements on

the sperm surface. Elements of the envelope (virus membrane proteins, i.e. virus receptors) serve as a lock-type mechanism and permit anchoring of complete viruses (envelope protein with genome) to certain surface characteristics of the sperms (key). Such sperm-specific virus receptors may be incorporated in the envelopes (particularly membrane-type envelopes) to serve as binding molecules for the sperms. Similar receptors are encountered in various microorganisms such as certain mycoplasms and bacteria. Covalent bonding of sperm-specific molecules such as antibodies and lectins, to components of the envelopes is possible , too. These molecules serve the same functions in binding as the above mentioned virus receptors. Blends of different binding molecules may also be used.

(b) Indirect binding of envelopes (vesicles or granulae) to sperms by means of mediators.

Mediators, a sort of adhesive, bind to specific structural elements on the sperm surface as well as to structural elements on the envelopes. The adhesive as such may comprise either two or more "locks", or a "key" plus one or more "locks".

The variety of possible indirect binding forms is much more complex than in the case of direct binding.

A simple form of binding may be obtained with positively charged ions ($Fe^{++}$, $Fe^{+++}$, $Zn^{++}$, and many others) located between negatively charged surfaces of sperms and envelopes. Of disadvantage may be certain instabilities due to the unspecific nature of binding by charge and probably due to chelate complex-forming substances that frequently neutralize the charged ions.

Another unspecific but very stable form of binding is achieved with substances that promote fusion of the membranes (spermal plasma membrane and lipid-containing envelope). However, the polyethylene glycol (PEG) used here more or less seriously with sperm mobility through agglutination or inhibition of tail movement. Certain cell-adhesive glycoproteins (fibronectins) also proved capable of unspecifically binding sperms to envelopes, but similar agglutination phenomena were observed with such glycoproteins.

None of the above problems is encountered on use of specific bridge binders such as lectins (phytoagglutinins, haemagglutinins, or invertebrate agglutinins). The most commonly used plant lectins are characterized by highly specific binding capacity and sometimes extremely high binding affinity for certain sugar molecules. Hardly ever is there recognized a group other than the terminal sugar group of an oligo- or polysaccharide chain which, in turn, is covalently bonded to a lipid molecule (glycolipid) or a protein molecule (glycoprotein). Some lectins always bind to a certain type of sugar molecule under the chosen conditions while others are capable of binding different types of sugars. As the lectin molecule has two or more binding sites (bivalent or polyvalent), lectins are capable of adhesively connecting surfaces showing identical terminal sugar groups. The strength of the binding between sperm surface and casing depends on the number of terminal sugar groups on the surfaces to be connected, i.e. on the density of the sugar molecules, on the number of available binding sites (valency) and the binding strength (affinity) of the lectins, provided lectins are added in excess. Due to the large quantity of terminal alpha-mannose (and alpha-glucose, respectively, for both types of sugar are bound by concanavalin A = Con A) on the sperms of many higher vertebrates, use of Con A lectin offers itself as particularly interesting. Oversaturation of alpha-mannose-carrying lipid membranes(envelopes) with Con A proved to be a simple procedure wherein, due to the amount of Con A added, each sugar residue is theoretically occupied by only one Con A-molecule. Thus, a large number of "free" mannose binding sites of polyvalent Con A will protrude from the surface of the envelopes. When such Con A-coated (actively binding) envelopes are added to sperms, they bind with their still free bindings sites of the Con A-molecules to the alpha-mannose residues on the sperm surface; the envelopes will then firmly adhere to the sperms. A large excess of actively binding envelopes prevents agglutination of the sperms as the entire sperm surface is saturated with envelopes.

The above described activation of binding capacity may also be applied to sperms. Incubation of sperms (in diluted suspension) with an excess of Con A results in saturation of all Con A-binding

sites on the sperm membrane. The surface of the sperms is subsequently covered by a large number of protruding free sugar binding sites through which any envelope showing the corresponding sugar groups (alpha-mannose and glucose, respectively) on its surface may be bound to the sperms.

Another type of binding molecules, namely antibodies, are, due to their bivalent or polyvalent properties,equally suited for the above technique of oversaturating the envelopes and subsequently binding them to sperms.Use of spermal antigen-specific antibodies requires the presence of spermal antigen on the envelope surfaces. This may most easily be accomplished by isolating the plasma membranes of sperms and using them as envelopes. Spermal antigen-specific antibodies and antisera, respectively, may be recovered from the serum of female animals after repeated immunization with washed sperms. Optimum binding capacity must be attributed to spermal antigen-specific, high affinity monoclonal IgM antibodies as they have ten binding sites (decavalent). Binding strength may approach that of covalent bonding. With today's gene-technological methods, preparation of so-called hybrid antibodies is possible, too. In contrast to naturally occuring antibodies, they may have two or more binding sites for recognizing and binding different structural elements. Thus, envelopes may be bound to sperms even if the structural surface elements of envelopes and sperms are not identical. Similar "unequal" binding is possible with a protein (protein A) found in the cell walls of Staphylococcus aureus and normal spermal antigen-specific antibodies. Protein A is capable of specifically binding with high affinity to that part of an antibody that does not bind antigen ($F_c$ part). When protein A is bound to membrane-inherent proteins of the envelope and spermal antigen-specific antibodies are subsequently offered to the envelopes thus loaded with protein A, the antibodies will bind with their $F_c$ parts to the envelopes, thus "extending" their sperm-specific antigen-binding elements outwards. Envelopes thus coated with protein A and antibodies are capable of binding very specifically and firmly to sperms.

Another phenomenon based on non-specific adsorption of proteins on surfaces may be utilized for forming very strong specific bonds between envelopes and sperm surfaces. This so-called "coating" (covering) of surfaces is made possible by the high non-specific binding capacity of proteins for certain surfaces such as cell membranes, synthetics or glass. Particularly suited for such "coating" procedures are e.g. antibodies. In the method of the invention, sperm-specific antibodies are used for covering the envelopes (vesicles, especially liposomes). "Coating" may be effected with spermal antigen-specific monoclonal antibodies, purified polyclonal antibodies or with an easily prepared high-titer antiserum. Envelopes covered with such specific binding molecules show the following advantages:

(a) simple and rapid preparation of actively binding envelopes,

(b) high stability of the envelopes, e.g. no auto-agglutination,

(c) with high-titer antibodies or antisera, no previous purification of the actual sperm-specific antibodies is necessary, and

(d) very high binding capacity for sperms.

For direct as well as indirect binding of envelopes to sperms, the specific binding structures may be stabilized by adding bond-promoting molecules (e.g. albumin, transferrin).

The envelopes of the substances to be transferred by sperms protect their contents from enzymatic or other modification and bind them to the sperms. The term "envelope" refers to any system which either entraps the substances to be transferred or is capable of forming aggregates therewith.

Preferably used as envelopes are vesicular structures, the substances to be transferred being either contained in the lumen thereof as an aqueous suspension or, if they are lipophilic, integrated in the walls of these vesicles.

The walls of such vesicles may consist of lipids or lipid-type materials that show high stability in aqueous media.

Suited for aggregation are materials permitting direct addition (without lumen) to the substances to be transferred without denaturing these substances. With the aid of calcium phosphate or histone protein, granulae may thus be prepared from e.g. nucleic acids.

Envelopes of the above type must meet the following requirements:

(a) They must consist of molecules capable of entrapping other substances without impairing them.

(b) Their surfaces must show structural elements that permit binding to the surfaces of sperms.

(c) The binding form must not negatively affect the ability of the sperms (or parts thereof) to fuse with the target cells.

(d) Their size must be a mere fraction of the size of the respective sperms.

(e) They must be capable of decomposing within the target cell, due to enzymatic degradation or the like, so as to release the transferred substances.

The wide variety of envelopes meeting the above requirements will be shown below.

As mentioned before, the surfaces of some microorganisms and viruses are provided with receptors permitting specific binding to sperms. A virus specialized on sperms of fish infects the egg cells by becoming attached to the sperm surface, thus finding its obligatory host. As virus membranes or cell walls of microorganisms are in themselves a sort of envelope, they seem perfectly suited for any transfer method involving sperms. But they can take up only a limited number of substances and are technically more difficult to handle (isolation, reaggregation of components) than the natural or artificial lipid membranes described hereinafter. Certain receptors from the membranes of viruses or microorganisms may be integrated into lipid membranes, serving therein as structural elements of specific binding affinity for sperms (virus receptors, protein A and the like).

Lipid membranes may be recovered without problems from substantially any animal cell. For the method of the invention, the only important aspect is that the selected cells or plasma membranes show surface characteristics that permit direct or indirect binding to the surface of sperms. This depends primarily on the desired form of binding.Sugar-binding lectins, for instance, may serve as mediator molecules between sperms and lipid membrane, and erythrocyte membranes (so-called "ghosts") may be used in this case as an easily obtainable starting substance as they exhibit a plurality of protruding sugar groups. Here too,the composition of the outwards extending structural elements of the lipid membrane may be modified so as to create new or better conditions for the desired form of binding between sperms and envelopes. Terminal sialinic acid molecules may be removed by cleavage, the lipid vesicles being incubated (before or after the substances to be transferred have been entrapped therein) with neuraminidase to effect cleavage. The resulting reduction in negative surface charge as well as the many previously hidden sugar molecules thus exposed offer new ways of binding the vesicles to sperms. Another possibility of optimizing special binding forms is use of enzymes capable of cleaving sugars as well as proteins. Even "recoating" of the lipid membrane surface may be accomplished by enzymatic treatment methods. Of particular interest in this respect are glycosyltransferases which covalently bond certain sugar molecules to the sugar backbone of the lipid membrane, thus making other forms of binding possible.

According to a further embodiment, sperm-specific antibodies are used as binding mediators; best suited for this purpose are envelopes prepared from plasma membranes of sperms. Due to identical antigenic structural elements on sperms and envelopes, the two units can easily be connected to each other through bivalent or polyvalent antibodies. The biochemical preparation method of such biological membranes prevents transfer of undesirable substances such as common viruses.

Lipid membranes of the above type may also be set up artificially from the respective lipids. Molecules (cerebrosides, glycoproteins and the like) must however be incorporated in the lipid mixture, their structural elements protruding from the surface permitting direct or indirect binding to sperms.

Other organic and/or inorganic substances are also suited for entrapping the substances to be transferred. A form of granular envelope, particularly suited for nucleic acids, is obtained by calcium phosphate precipitation of DNA. What physico-chemical properties actually make binding of calcium phosphate-precipitated DNA to cell membranes possible is not yet fully understood, but the method as such is practicable.

A natural type of granular envelope for DNA of higher organisms is obtained by binding DNA to histone proteins. The histone envelope serves a stabilizing function and provides some protection from degradation to the DNA. However, for improved binding to sperm surfaces, the histone-associated DNA is advantageously incorporated in vesicles as they show far better binding characteristics and offer added protection.

What type of substance shall be transferred to the target cells depends on the specific purpose of such transfer. The method of the invention may serve three objects:

(a) production of transgenic organisms or transgenic cells;

(b) use as a test system for determining the effects of substances in somatic cells and egg cells in particular;

(c) use as a technique for studying embryogenesis and teratology in vivo and in vitro.

Depending on the object, a plurality of substances may be transferred according to the method of the invention. Due to the proposed technique of simultaneously preparing envelopes and enclosing therein the substances to be transferred, the most diverse substances may be entrapped in the same manner and without the need for specially developed processing steps. Binding

to sperms and thus transfer to the target cells is not dependent either on the type of substance within the envelopes.

Transgenic organisms or transgenic cells can be obtained only by transferring genetic material in form of nucleic acids. A plurality of different genes useful for improving production are available at present or will become available in the near future. Of particular interest are: the gene for somatotropin (growth hormone), genes for various milk proteins (caseins), use of anti-sense DNA for controlling specific diseases, and use of genes for producing vaccines in large quantities (genetic farming).

The invention makes use of the phenomena of natural fertilization (fusion of sperms and eggs) and of artificial insemination procedures (manipulative transfer of sperm) to provide a significantly simplified method of transferring genes to organisms wherein conventional techniques such as embryo flushing, micromanipulation, surgery and transfer of embryos are avoided.

The procedure by which the desired substances (genes) are prepared is substantially the same as in prior methods of producing transgenic organisms. Recommended is use of special integration and expression enhancers (metallothioneine promoters, long terminal repeats) to facilitate integration and expression of the genes thus transferred. Nucleic acids (genes) in linear or circular form are either entrapped in artificial vesicles or processed with specific proteins and inorganic salts, respectively, to form granulae.

Prior to actual use in medicine or in places where people are working, many substances must be tested for harmlessness to the human health. In the last years, public criticism of experiments with animals has been steadily increasing so that the authorities of several countries now demand alternatives to the use of animals in the necessary preliminary tests.

According to the method of the invention, a multitude of substances may now be tested for potential pathogenic effects directly on somatic cells, particularly on egg cells and the first embryonal stages, the previously employed techniques such as fusion or micro-injection with their strong side effects being avoided in this manner.Tested by the method of the invention may be e.g. amino acids, porphyrin or antibiotics.

To facilitate verification of the successful transfer of such minute quantities of practically undetectable substances, so-called "tracers"may be added and transferred within the same envelopes. Illustrative of such tracers are small genome units that undergo self-replication in the course of embryonal development (episomal Bovina papilloma virus and many others); presence of such units in embryos is relatively easy to verify by means of DNA hybrid-ization techniques. Testing in the above manner may be conducted not only in vitro but in vivo as well without involving the organism of the foster mother.Enzymatic transformation of many substances by the maternal organism frequently prevents in vivo investigation of embryopathogenesis.

Many phenomena of gene regulation in multicellular organisms cannot be adequately investigated with today's techniques. The proposed method wherein sperms are used for transferring gene-regulative substances such as peptide hormones, steroid hormones, certain forms of nucleic acids (viruses, enhancers, anti-sense RNA) to egg cells or somatic cells avoids the disturbing side effects of presently practiced methods and offers a greatly facilitated technique. Even studies on gene regulation, embryogenesis, organogenesis and teratology, in vivo as well as in vitro, are more simple technically when conducted according to the invention.

In principle, two types of cells may serve as target cells for the substance-loaded sperms: the natural target cells of sperms, i.e. egg cells, and all other cell types of multicellular organisms, including transformed cells such as teratocarcinoma cells.

Egg cells of many invertebrates, fish and amphibia are extracorporally accessible (spawning). The egg cells of most higher vertebrates lie more or less hidden in the primary reproductive organs (sexual organs) of the female. They are accessible only by surgical means. Sperms alone are capable of reaching the egg cells within the sexual organs of the female and transferring (aided by the method of the invention) additional substances to these egg cells. Bovine sperm covers the long distance between the site of sperm deposition (natural as well as artificial insemination) near the cervix and the ovarial end of the fallopian tube within a few minutes. The slow proper motion of the sperms (0.2 mm/sec.) is strongly enhanced by ciliary motion and the activity of secretory fluids within the female genital tract. The egg cells of all higher vertebrates (intracorporal insemination) are covered by a protective layer of nutrient cells, the so-called Corona radiata, and an inner mucopolysaccharide layer, the so-called Zona pellucida. Lysosomal enzymes contained in the spermal acrosome enable the sperms to penetrate the protective layers and fuse with the egg cell. In this so-called acrosome reaction, sperms lose their foremost head portion of the outer plasma membrane but retain the rear portion of the plasma membrane with any substance adhering thereto until they have reached the cytoplasm of the egg cell. For in vitro fertilization, the protective layers surrounding the egg cells are often removed by physicochemical means before sperms are added.

In vitro, i.e. under artificially created conditions, sperms may even enter into somatic cells (Brackett et al., 1971: PNAS 68(2): 353).

A further use of the method according to the invention is thus possible, namely transfer of substances to somatic cells and transformed embryonal cells, e.g. with the object of conducting somatic gene therapy.

The advantages of the method according to the invention reside in the simplicity of sperm treatment: sperms need not necessarily

undergo endocytosis with vesicles or granulae and, thus, with the substances to be transferred but may carry them "pick-a-back" on the spermal membrane into the egg cell or somatic cell where the transferred substances become effective.

No special equipment is required for producing transgenic animals according to the method of the invention. Needed is a vessel for the sperms into which a blend of vesicles or granulae and buffer is then added from a second (easily carried and shipped) container. Depending on the desired form of binding, addition of a "neutralizing" solution may be necessary so as to saturate surplus binding sites on the vesicle-loaded sperms. After an appropriate incubation period, the blend is used for inseminating oestric animals (in vivo, intracorporal fertilization) or added to egg cells (in vitro, extracorporal fertilization). In the method of the invention, each sperm becomes a vehicle for vesicles or granulae. The number of transgenic descendants actually obtained therefore depends on the rate of inclusion (percentage of DNA-containing vesicles bound per sperm unit) and the rate of natural fertilization (successful pregnancy after insemination).

Especially when transgenic domestic animals such as cattle, sheep and the like are to be produced in accordance with the method of the invention, established animal breeding organizations may be involved. Most important by far for the method of the invention are the insemination centers where practically all sperm specimens for inseminating female animals and producing the entire descendant generation are obtained.

To bring about improvements in each new animal generation (the only useful object of breeding), breeders presently rely on selection of optimum sires which have to pass a series of time-consuming efficiency tests. Annual advance in breeding depends not only on strict selection but also on the respective generation interval which is about 5 to 7 years for cattle and about 2 years for sheep and pigs. Firmly establishing a new characteristic (trait , gene) in an entire population by cross-breeding is a

long-term venture since very few animals are suited for the parent generation. Factors such as original number of parent animals, generation interval and strictness of selection may retard introduction of new genes into an entire population when the hitherto accepted breeding methods are employed. Moreover, a number of useless or even harmful genes will be passed on to the next generation together with the desired gene that was the reason for cross-breeding or selection.

Another advantage of the method according to the invention is the elimination of many disturbing factors. First of all, it is now possible to transfer a s i n g l e gene without carrying along other useless or undesirable genes as is the case in cross-breeding. Secondly, the selected gene may be introduced at one and the same time into all descendants of a population. For this purpose, one merely has to treat all spermal specimens with the gene-containing vesicles (or granulae). Presence of the transferred gene in the organisms of the descendant generation is easily verifiable (e.g. by taking blood samples) so that, thirdly, the generation interval is reduced as the animals may be used in breeding at a very early stage. A multitude of genes is available which should, of course, not be introduced at one and the same time; suitably, a single gene (or a small number of genes) per animal is introduced during the course of natural fertilization. This procedure is particularly preferred for experiments wherein individual (new) genes are tested for specific effects, efficiency or effects in various positions.

The method of the invention is ideally suited for today's breeding techniques. In special cases, e.g. when embryos are to be exported or imported, the low transportation costs of embryos treated in accordance with the invention are of particular advantage. For this case, pre-ovulatory eggs may be fertilized in vitro with substance-loaded sperm and, after successful development to the morula stage, subjected to deep-freezing or short-term preservation; they may then be shipped as transgenic embryos to any desired place at low cost.

Transgenic animals are required in research, too, for investigating specific questions. As artificial insemination of laboratory animals is easily accomplished, the method of the invention is perfectly suited for laboratory practice as well as for studies on fertilization in vitro. Working in accordance with the method of the invention requires less time and personnel than any of the traditional methods ~~of~~ (embryo and) gene transfer.

EXAMPLE 1

(A) Recovery and concentration of genes.

In principle, a structural gene may be recovered as cDNA by means of RNA-dependent DNA polymerase (reverse transcriptase) from tissue-isolated messenger-RNA. The cDNA thus obtained permits identification and isolation of genomic DNA, i.e. the natural gene. Recovery of genomic DNA fragments is state of the art (general description: Cooper, T.G. 1981: Biochemische Arbeitsmethoden, Walter de Gruyter, Berlin; Old, R.W., Pimrose, S.B., 1981: Principles of Gene Manipulation, Blackwell, Oxford; Maniatis et al., 1982: Molecular Cloning, Cold Spring Harbor).

A multitude of known DNA fragments from a variety of species are available at present in so-called gene banks (depositories). Also available are more than 4,000 genes of determined sequence derived from a variety of organisms, starting from SV 40-DNA up to the casein gene of cattle. The individual genes comprise up to several thousands of base pairs (kb). In gene analysis, a chart of restriction enzymes is normally compiled, either empirically through all possible endonucleases or, if the nucleotide sequence is known, by tetra- or hexanucleotide comparison with any known restriction enzyme (data information by the European Molecular Biology Laboratory, Heidelberg).

Any structural gene may be linked with a promoter of choice (to improve gene expression). Promoter is called that part of a gene that induces transcription.

The structural gene of the growth hormone, for instance, is positioned between two Bam HI-recognition sites for restriction enzymes. The Bam HI-fragment is isolated by digesting 100 µg DNA of the plasmide containing the gene for the growth hormone with 100 units of Bam HI (37°C, two hours). Digestion reaction is terminated by adding a stopper solution (Maniatis et al., 1982: Molecular Cloning, Cold Spring Harbor), the mixture being then applied to 0.7 % agarose gel. The DNA fragments produced by the enzyme are electrophoretically separated according to size, eluted from the gel and purified with phenol/chloroform. DNA concentration is photometrically determined ($OD_{260}$ x 50 x dilution factor = µg/ml). In the same manner, a promoter (metallothioneine) commercially available in a plasmide pMMT 342 is isolated.

The metallothioneine promoter (MT) lies within an Eco RI - Bgl II fragment. Once the fragment is present in pure form (see above), it is ligated with the structural gene (method: Maniatis et al., 1982: Molecular Cloning, Cold Spring Harbor). Ligation is possible with minute quantities. To obtain larger quantities of the new material set up in this manner, promoter and structural gene are incorporated (ligated) in a plasmide which, after transformation in bacteria, permits practically unlimited propagation. A well known plasmide is pBR 322, the prototype of many presently used plasmides. It comprises various essential functional elements: "origin of replication (Ori)" and two genes coding for resistance to antibiotics (ampicillin, tetracycline). Such plasmides are known to have various sites where restriction enzymes may cut. Used to an ever increasing extent are the so-called polylinkers which show a large number of recognition sites for restriction enzymes within a very short nucleotide sequence (50 bp), thus offering many possibilities of recombination. Polylinker pSP 64 and, as a modification thereof, polylinker pSP 65 have been used successfully for such purposes.

Plasmides replicate extrachromosomally in bacteria, thus replicating the inserted DNA sequence, too, so that cloning of the desired gene to obtain larger quantities thereof (100 to 1,000 ug per batch) is possible.

The MT-structural gene fragment is ligated into polylinker pSP 65, the fragment being present in the form of Eco RI - Bgl II/Bam HI-Bam HI. Polylinker pSP 65 also has one Eco RI- and one Bam HI-site; opening of these sites results in linearization of the plasmide. Two different cohesive ends corresponding to those of the fragment to be inserted have thus been created. Ligation produces two circular plasmides, namely the original plasmide through auto-ligation, and the desired plasmide having a size of 6.9 kb.

The above procedure is applicable to almost any gene in combination with any promoter or additional enhancer elements.

Many variations of setting up a modified gene (and not of using natural gene regulators in front of the structural gene) are conceivable in accordance with the above described method. Any structural gene and any promoter as well as additional enhancers or even a tandem configuration of various promoters may be used for improving the activity (transcription) of the structural gene as such.

As a rule, the gene is now present as part of a circular or linear plasmide. As such, it is dissolved in TE-buffer (0.01 M Tris, 0.001 M EDTA, 0.15 M PBS; pH 7.2) after having been purified in phenol/chloroform. For the subsequent steps of the method, concentration should be kept between 200 and 500 µg/ml, usually determined by photometer.

(B) Preparation of liposome components.

Sperms carry complete genomes within their DNA-protecting membranes so that the DNA never comes into contact with degrading substances on the rather long way to the egg cells. In the course of evolution, sperms became fairly reliable in taking the DNA compressed therein to the target cells. However, as DNA is so tightly packed within the sperms, any attempts at introducing additional genes into the sperms were bound to fail.

Additional DNA attached to the outer surface of sperms must therefore be protected if it is to safely reach the egg cells. Best suited for this purpose are artificially prepared vesicles in the form of liposomes. Liposomes are prepared from natural components of animal cells. Such components are commercially available in form of phospholipids (lecithin, phosphatidylethanolamine and the like). For optimum preparation of liposomes, additional stabilizers such as cholesterol, phosphatidylserine or the like are used. A simple way of obtaining the essential membrane components is use of so-called "ghosts", i.e. fat-soluble membrane fragments of erythrocytes. For this purpose, blood of rabbits in native stage is treated with 0.15 % sodium heparin (about 50 ml per batch). The fresh blood is centrifuged at 3,000 rpm, the plasma being decanted. The procedure is repeated twice under addition of 0.15 M PBS to wash the erythrocytes, the leucocytes in the interphase being discarded.

The cells are then subjected to lysis in distilled water with 0.1 % Triton 100 over night to break open the cell membrane and dissolve the cell contents (haemoglobin). By centrifuging three times at 20,000 g (4°C), the membrane fragments are separated from the haemoglobin-containing supernatant. The pellet is then taken up in a chloroform/methanol mixture (20 ml, 5:1) and mixed therewith over night under gentle agitation. The liquid molecules are thus dissolved in chloroform while hydrophilic substances remain in the aqueous phase. The mixture is then centrifuged at 3,000 rpm. The upper phase contains water-soluble components, the interphase amphiphilic substances, and the lower (chloroform) phase the lipoid components, e.g. phosphatidylcholine, cholesterol, and glycolipids constituting the binding-specific epitopes of the prospective liposomes. Concentration of the main components may be determined by high-pressure liquid chromatography (HPLC) but such determination is unneccessary when preparation is performed under standardized conditions.

Of the recovered lipid/chloroform solution, 4 ml are used in the subsequent preparation of liposomes. Required is a 25 ml round-

bottom flask wherein chloroform is evaporated at about 30°C by means of a rotary evaporator or an aspirator.

The residue, a highly viscous material composed of natural lipids of the erythrocyte membrane is vented with nitrogen. The lipids are then dissolved in 1 ml of diethylether.

According to one of the many other embodiments according to the invention, all components may be acquired commercially, a useful combination being e.g. phosphatidylcholine, cholesterol, phosphatidylserine and a glycolipid having a terminal alpha-mannose group. The above components are mixed in a ratio of 5:1:3:2 and evaporated as described before.

Very stable and cell-compatible liposomes are obtained by using phosphatidylcholine and phosphatidylethanolamine in a ratio of 4:1. These compositions are particularly suited for the detergent method described below.

Membranes of sperms may be used according to a further embodiment. Sperms freed of seminal plasma are treated under constant agitation with a chloroform/methanol mixture (5:1, v/v) to dissolve the lipid components of the membranes. Centrifuging at 3,000 rpm separates the chloroform phase containing the desired components from the other components. The purpose of this procedure is recovery of all required natural membrane components including the very important epitopes that permit specific binding.

(C) Entrappment of DNA in liposomes.

After the membrane-forming components have been dissolved in diethylether, preparation of liposomes under simultaneous entrapping DNA or other molecules is started. The form of DNA (circular or linear) is not important, Inclusion rate rather depends on the concentration of DNA in the aqueous solution, the preparation of which has been described above. As will be understood, the few femtoliters (fl) randomly taken (encapsulation) from the aqueous solution contain the more dissolved molecules,

the higher the concentration of dissolved material. To form liposomes and enclose therein aqueous volumina, DNA dissolved in a 500 µl volume (100 µg DNA in 200 µl TE-buffer and 300 µl PBS) is now added. Two phases are formed in this manner, a lower aqueous phase and an upper ether phase. The two phases are emulsified on a vortex agitator (2 to 5 min., 30°C) until a fine emulsion is obtained. Due to energetics, spherical uni- or multilamellar structures form of the lipoid substances while ether evaporates in part. The vesicles thus forming enclose a small volume of the aqueous phase whereby dissolved DNA fragments are taken up in the interior of the liposomes.The spherical form of the membrane components remains stable unless attacked by organic solvents. For that reason, residual ether must be removed as fast as possible from the emulsion. This is accomplished by immediate evaporation with the aid of a rotary evaporator or an aspirator under gentle shaking (20 min.).

Also suited for liposome preparation is the detergent method. Here too, fat-like components are evaporated in a round-bottom flask by means of a rotary evaporator. In contrast to the ether method, a detergent (sodium cholate) is admixed (20 µg at a phospholipid content of 20 to 50 µg) before chloroform is evaporated.

After evaporation of chloroform, a fatty layer composed e.g. of 50 µg lecithin (phosphatidylcholine), cholesterol, natural cell components and sodium cholate (ratio 5:1:2:3) remains on the inner surface of the flask. By addition of 1 ml PBS with 300 µg DNA are the fat-like substances dissolved ( sodium cholate). The volume (1 ml) is then dialyzed against PBS under cautious agitation for 3 hours, and the liposomes thus formed entrap minute volumes of DNA-containing aqueous solution.

The artificial vesicles thus obtained have surfaces similar to those of erythrocytes or sperms; in any case, their surfaces show epitopes that permit binding to sperms while their interior contains an aqueous phase comprising dissolved DNA. As liposomes

are impermeable to macromolecular substances, the entrapped DNA is perfectly protected from degradation (e.g. DNases). Any other molecule may be entrapped in liposomes in the same manner. The liposomes are recovered by centrifugation at 40,000 g and taken up in PBS. The ratio pellet to solvent may be from 1:5 to 1:10. PBS may be replaced with other buffer solutions as long as the pH is between 6.9 and 7.3 and a physiological concentration of 0.15 M is retained (with cells of mammals as target cells). An example of suitable buffer solution is the so-called Ringer solution which has the following composition: 0.9 % NaCl (100 ml), 1.15 % KCl (4 ml), 2.1 % $KH_2PO_4$ (1 ml), 3.82 % $MgSO_4 7 H_2O$ (1 ml), 1.3 % $NaHCO_3$ (2 ml, $CO_2$-saturated). To improve energy supply of the sperms (motility), 0.5 % of fructose are added to the Ringer solution.

(D) Adsorption of Sperms.

The DNA to be transferred (one or more functional groups of any type desired), i.e. several copies thereof in an aqueous solution (the number of copies depending on the concentration of DNA in the solution), is entrapped within a defined structure (vesicle or granula) that has surface-protruding structural elements (epitopes) capable of establishing close contact with certain binding molecules (lectins or antibodies). The epitopes of liposomes are sterically identical or very similar to those on the surface of sperms; however, when unspecific binding forms ("coating") are employed, they may be of entirely different configuration or missing completely.

Sperms are recovered artificially by methods well described for domestic and laboratory animals (Paufler, S.K., 1974: Künstliche Besamung I, Schaper, Hannover). Seminal plasma is removed from the ejaculate by centrifugation (400 rpm) and buffer solution (Ringer solution with 0.5 % fructose) is added. In practice, the collected sperm (several billions of sperms per ejaculate) is diluted and divided into portions; depending on the species, there may be obtained from 20 (rabbit) to 100 (bull) insemination specimen per ejaculate.

It is important for the method of the invention that the diluent used is free of molecules which are capable of interacting with the respective binding molecules.

Before the sperms are mixed with liposomes, binding affinity of the liposomes for sperms must be established. An excess of concanavalin A (500 µl + 50 µl Con A (50 mM)) is therefore added to the liposomes. The specific epitopes are thus saturated with Con A as schematically shown in Figure 2.

Due to the excess in lectin, all epitopes on the liposome surface are occupied; agglutination of liposomes which would be inevitable at lower lectin concentrations is thus prevented. Lectin is added to the liposomes at room temperature. Under thorough mixing, saturation is complete within 30 minutes.

To increase the number of actively binding sugar molecules on the liposomes prepared from components of the cell membrane, 10 µl of neuraminidase (1 mg/ml, Boehringer, Mannheim) are added to the liposome dispersion and incubated therewith for 20 minutes at 37°C. After washing with PBS and 0.5 % BSA, the pellet is taken up in PBS. Terminal sialinic acid groups are removed in this manner to expose the sugar molecules.

A further possibility of binding liposomes to sperms is use of sperm-specific antibodies. To produce antibodies, female animals (rabbits) of an age of 3 to 5 months are subcutaneously immunized with an ejaculate free of seminal plasma (incomplete Freund's adjuvant). Second immunization takes place after two weeks and third immunization after an additional week. Four days later, blood (10 to 20 ml) is taken from the immunized animals, cooled and centrifuged at 3,000 rpm to recover the serum. Contained in the serum are (polyclonal) antibodies against epitopes on the spermal surface. Antisera thus obtained cause agglutination of rabbit blood even when diluted in a ratio of 1:1,000.

Spermal antigen-specific antibodies permit two forms of binding between envelopes and sperms. In the first case, the envelope components are obtained from spermal membranes and the envelopes are prepared in the above described manner. Bivalent or polyvalent antibodies then bind identical or similar structural elements on the surfaces of envelopes and sperms.

In the second case, unspecific adhesion of antibodies is utilized for covering the envelope surface with antibodies (coating). Spermal antigen-specific binding sites of the antibodies will protrude from the envelope surface and permit binding to sperms.

In a given case, 0.5 ml of antiserum diluted in a ratio of 1 : 50 with Ringer solution (spermal agglutination titer at dilution of 1:1,000) are added to 0.5 ml of liposome suspension and incubated over night at 4°C. Excess antibodies are washed (2 ml of Ringer solution) from the mixture by means of a fine-pore filter (millipores, 0.2 µm) and the purified liposomes on the filter are taken up in 0.5 ml of Ringer solution (counter-current flow). The liposome suspension is centrifuged for 2 minutes at 12,000 rpm whereby large unsuited liposomes are precipitated in the pellet while liposomes of the desired size (200 to 800 µm) remain in the supernatant.

Through free lectin and antibody binding sites, respectively, the liposomes are then bound to the surfaces of sperms. Liposomes

and sperms are cautiously mixed at room temperature so that adsorption of the binding molecule-loaded liposomes on the specific epitopes of the sperm surface is completed within 20 minutes.

Sperms carrying large adsorbed liposomes are recognizable under a light microscope by stable (not even removable by washing) globular forms adhering thereto. Ethidiumbromide-containing DNA was entrapped in liposomes and adsorbed on sperms. Ethidiumbromide in aqueous solution may be entrapped in liposomes, too, the liposomes then showing a pink glow under u.v. light. Ethidiumbromide binds (intercalation) specifically to DNA which is then discernible in the liposomes by its green color. Adsorbed on sperms, it produces a green fringe around the sperms.

When lectin-coated envelopes are bound to sperms, a large number of unsaturated lectin binding sites will protrude from the surface. To prevent adhesion of the sperms on sugar-coated surfaces within the female genital tract (immobilization), the surplus lectin binding sites (Con A)are best saturated just prior to insemination by adding 10 to 50 µl of alpha-mannose or alpha-glucose (0.01 M). The exact quantity of sugar to be added depends on the number of envelopes, the respective type of lectin and the sperms and may be determined for each system by simple preliminary tests.

(E) Artificial insemination.

Once the liposomes are bound to the sperms, the sperm specimen is ready for artificial insemination procedures. With rabbits, ovulation must first be initiated by intravenously administering a gonadotropic hormone (LH = luteinizing hormone; HCG = human chorion gonadotropin). Immediately following the injection, a sperm specimen is deposited into the vagina with an insemination pipette. The sperms migrate upwards through the female genital tract to arrive at the fertilization site (ampulla) where they enter the freshly ovulated eggs.

Together with the sperm, the only mechanism capable of penetrating all of the membranes covering the egg cell, the gene copies come into the region of the amphimictic nucleus where the diploid chromosome set is formed.

Artificial insemination of spontaneously ovulating species such as cattle, horses, pigs and sheep is effected in accordance with the natural oestrus of the animal.

(F) Verification of DNA integration.

When the liposomes carried by sperms have reached the interior of the egg cell, they undergo lysis with cell-inherent enzymes. DNA thus released is capable of integrating in the genome. Successful integration into the embryo is verified by in situ hybridization. For this purpose, the (transgenic) embryos (bicellular stage to blastocyst stage) are washed out of the fallopian tube or uterus of the rabbit (by surgical means in this particular case) and are then immobilized on an object slide by a fixing liquid (methanol/glacial acetic acid, 3:1, v/v).

Object slide and blastocysts fixed thereto are heated to 90°C to effect denaturation of complementary DNA strands of the chromosomes. Hybridization is conducted with a radioactively labelled or biotin-labelled probe (in this particular case, with the gene for the growth hormone). As for the techniques of in situ hybridization, reference is made to related literature (Gall and Pardue, 1969: PNAS 63: 378-383; and others).

EXAMPLE 2

Transporting Non-Nucleic Acids by Liposomes into Egg Cells.

To demonstrate the possibilities of transport by liposome-loaded sperms, the enzyme pronase and colcemide (cell cleavage inhibitor) were transferred to egg cells of rabbits. Both substances have a negative effect on the development of the zygote, inhibiting essential stages of cleavage (spindle formation) and, most of all, cell metabolism (by proteolysis). Egg cells treated with these substances remain in the monocellular stage as zygotes, while zygotes fertilized with sperm that was loaded with substance-free ("empty") liposomes show normal development.

(A) Preparation of liposomes.

Used as components of the liposome envelopes are phosphatidylcholine (8 mg) and gangliosides (2 mg), sodium cholate (6 mg) being added as a detergent. Phosphatidylcholine and the gangliosides are dissolved in methanol and blended in the respective volumes in a glass flask. Sodium cholate is weighed in as a powder. After thorough mixing, methanol is evaporated by means of a rotary evaporator or an aspirator. An opalescent layer thus forms on the inner surface of the flask.

(B) Entrappment of the substances to be transferred.

To the solid phase on the inner surface of the flask an aqueous solution of substances is added to be transferred. Concentration of pronase may be 100 µg/ml PBS (phosphate buffered saline), concentration of colcemide e.g. 50 µg/ml PBS. The total amount of aqueous solution added is 1 ml. Due to the presence of a detergent, the lipid-containing layer on the inner surface of the flask dissolves completely. The mixture is then dialyzed against PBS for 5 hours under constant agitation (membrane permeable for molecules having a molecular weight below 10,000 d). Sodium cholate evaporates, and spherical bodies (liposomes) containing the substances to be transferred are formed. Liposomes of varying size are obtained after dialysis, their average diameter being about 600 nm, i.e. below the visibility range of a light microscope; contained within these liposomes is a buffer solution of the substances to be transferred.

(C) Surface treatment of liposomes.

As the membranes of the liposomes show terminal sialinic acid groups, 20 µg/ml of neuraminidase are added to the liposomes to remove the sialinic acids of the gangliosides by cleavage. Surface charge (negatively charged sialinic acid) of the liposomes is thus reduced, and sugar molecules originally hidden under sialinic acids are exposed to serve as additional binding sites for lectins such as Con A and PNA. After 30 minutes of incubation with neuraminidase (37°C), Con A and PNA (10 µg/ml each) are added to the suspension. The terminal sugar groups on the liposomes are thus saturated with lectins (incubation 15 min. at room temperature).

(D) Calibration of liposomes.

The sperms are advantageously loaded with size-calibrated liposomes as oversized liposomes are not suited for transport through the various cell barriers (Zona pellucida and membrane of egg cell) while undersized liposomes, due to their small lumen, cannot take up adequate volumes of substances. The surface-treated liposomes are therefore filtered under moderate pressure (0.2 µm pore size of filter membrane). Undersized liposomes as well as free lectins and neuraminidase pass through the filter and are discarded. Rinsing the same filter from the reverse side results in a fraction comprising medium-sized as well as oversized liposomes. This fraction (about 1 ml) is centrifuged for 4 minutes at 12,000 g to pelletize oversized liposomes. Liposomes of the desired size remain in the supernatant.

(E) Adsorption on sperms.

The remaining surface-treated and calibrated liposome fraction is then added to sperms. Freshly ejaculated sperm of rabbits is centrifuged for 10 seconds at 3,000 g, the supernatant being discarded. The resulting sperm pellet is suspended in Ringer solution (0.5 ml). Liposomes are blended into the sperm suspension and incubated at room temperature for 30 minutes. During this period, the liposomes become bound to the sperms.

(F) Artificial insemination and verification of substances transferred into the egg cells.

Liposome-loaded sperm is now used for inseminating animals. After two days, the embryos are washed from the genital tract of the test animal and inspected under a microscope. Control embryos fertilized with sperm carrying "empty" liposomes show normal development, i.e. they reach a four-cell or eight-cell stage within a period of 48 hours after fertilization. Egg cells fertilized with sperms that have been loaded with pronase- or colcemide-containing liposomes are still in the zygote stage (i.e. a single cell which, in contrast to the unfertilized egg cell, shows two polar bodies, thus being identifiable under the microscope) after two days, some cells even showing phenomena of lysis.

Presence of two polar bodies in an otherwise undeveloped cell is clear evidence that the cell was successfully fertilized but inhibited in its development by pronase and colcemide transferred by the sperm.

CLAIMS:

1. A method of transferring organic and/or inorganic substance(s) to egg cells and/or somatic cells of animals, wherein sperms of the respective type, optionally modified by chemical or physical means, are combined with vesicles or granulae containing organic or inorganic substance(s) whereupon the resulting loaded sperms are contacted with egg cells or somatic cells under intracorporal or extracorporal conditions.

2. Method according to claim 1 wherein said vesicles or granulae consist of organic or inorganic substance(s) provided with envelopes.

3. Method according to claim 2 wherein said envelopes comprise:
(a) naturally occuring cell constituents,
(b) substances of viral and/or microbial origin, or
(c) (synthetic) inorganic or organic material.

4. Method according to claim 2 wherein said envelopes comprise one or more substances selected from the group consisting of lipids, phospholipids, glycolipids, isoprenoid lipids, lipoproteins, proteins, glycoproteins, saccharides and/or organic or inorganic salts.

5. Method according to claim 1 or 2 wherein said vesicles or granulae are bound to sperms by addition, fusion with the spermal membrane, or endocytosis.

6. Method according to claim 1 or 2 wherein said vesicles or granulae are bound to sperms by van der Waals' forces, hydrogen bonding, ionic bonding and/or covalent bonding.

7. Method according to claim 1 or 2 wherein said vesicles or granulae are bound to sperms by adding organic and/or inorganic substance(s).

8. Method according to claim 1 or 2 wherein said vesicles or granulae are bound to sperms by means of antibodies and antisera, respectively, lectins,agglutinins,viral, procaryotic or eucaryotic receptors or receptor-like molecules.

9. Method according to claim 1 or 2 wherein the substances to be transferred are selected from the group consisting of amino acids, peptides, proteins, glycoproteins, lipoproteins, coenzymes, metabolites, enzyme inhibitors, transcription or translation inhibitors, metabolism inhibitors, mitogens, nucleosides, nuclectides, nucleic acids, porphyrins, lipids, preferably phospholipids, glycolipids or isoprenoidlipids,monosaccharides, oligosaccharides,polysaccharides,glycosides, hormones, vitamins, pharmaceutic agents and inorganic substances, preferably metallic compounds.

10. Method according to claim 1 or 2 wherein the substances to be transferred are selected from the group consisting of nucleic acids, preferably DNA in linear or circular form, single- or double-stranded, as a heteroduplex, or RNA.

11. Method according to claim 1 or 2 wherein, depending on the target cell, there are used sperms of Metazoa, in particular of animals of the phylum Arthropoda, the subphylum Vertrebrata,the class of Osteichthyes, Aves or Mammalia, i.e. of the order of Perissodactyla, Artiodactyla, Lagomorpha, Rodentia, Carnivora or Primates, the suborder of Prosimiae, the superfamily of Ceboidea or Cercopithecoidea, the family of Hylobatidae or Pongidae.

12. A composition comprising sperms of animals and, bound thereto, vesicles or granulae containing organic or inorganic substance(s).

13. A composition according to claim 12 wherein said vesicles or granulae consist of organic or inorganic substance(s) provided with envelopes.

14. A composition according to claim 13 wherein said envelopes comprise:
(a) naturally occurring cell constituents,
(b) substances of viral and/or microbial origin, or
(c) (synthetic) inorganic or organic material.

15. A composition according to claim 13 wherein said envelopes comprise one or more substances selected from the group consisting of lipids, phospholipids, glycolipids, isoprenoid lipids, lipoproteins, proteins, glycoproteins, saccharides and/or organic or inorganic salts.

16. A composition according to claim 12 or 13 wherein the organic substance contained in said vesicles or granulae is selected from the group consisting of amino acids, peptides, proteins, glycoproteins, lipoproteins, coenzymes, metabolites, enzyme inhibitors, transcription or translation inhibitors, metabolism inhibitors, mitogens, nucleosides, nucleotides, nucleic acids,porphyrins, lipids, preferably phospholipids, glycolipids or isoprenoidlipids, monosaccarides, oligosaccharides, polysaccarides, glycosides, hormones, vitamins and pharmaceutic agents.

17. A composition according to claim 12 or 13 wherein the organic substance contained in said vesicles or granulae is selected from the group consisting of nucleic acids, preferably DNA in linear or circular form, single- or double-stranded, as a heteroduplex, or RNA.

18. A composition for use in the method according to any of claims 1 to 11 comprising in an aqueous medium substance(s) to be transferred and optionally material

suited for entrapping or binding said substance(s) to sperms, as well as preservatives and/or, in particular, buffers for adjusting the pH in a range of preferably 3 to 10 and total molarity at a value of 0.001 to 2.0.

19. A composition according to claim 18 wherein the substances to be transferred are selected from the group consisting of amino acids,peptides,proteins,glycoproteins,lipoproteins,coenzymes,metabolites,enzyme inhibitors,transcription or translation inhibitors,metabolism inhibitors, mitogens, nucleosides, nucleotides, nucleic acids,porphyrins, lipids, preferably phospholipids, glycolipids or isoprenoidlipids,monosaccharides, oligosaccharides,polysaccharides,glycosides, hormones, vitamins, pharmaceutic agents and inorganic substances, preferably metallic compounds.

20. Transgenic animals produced according to the method set forth in any of claims 1 to 11.

Prior Techniques of Producing Transgenic Animals

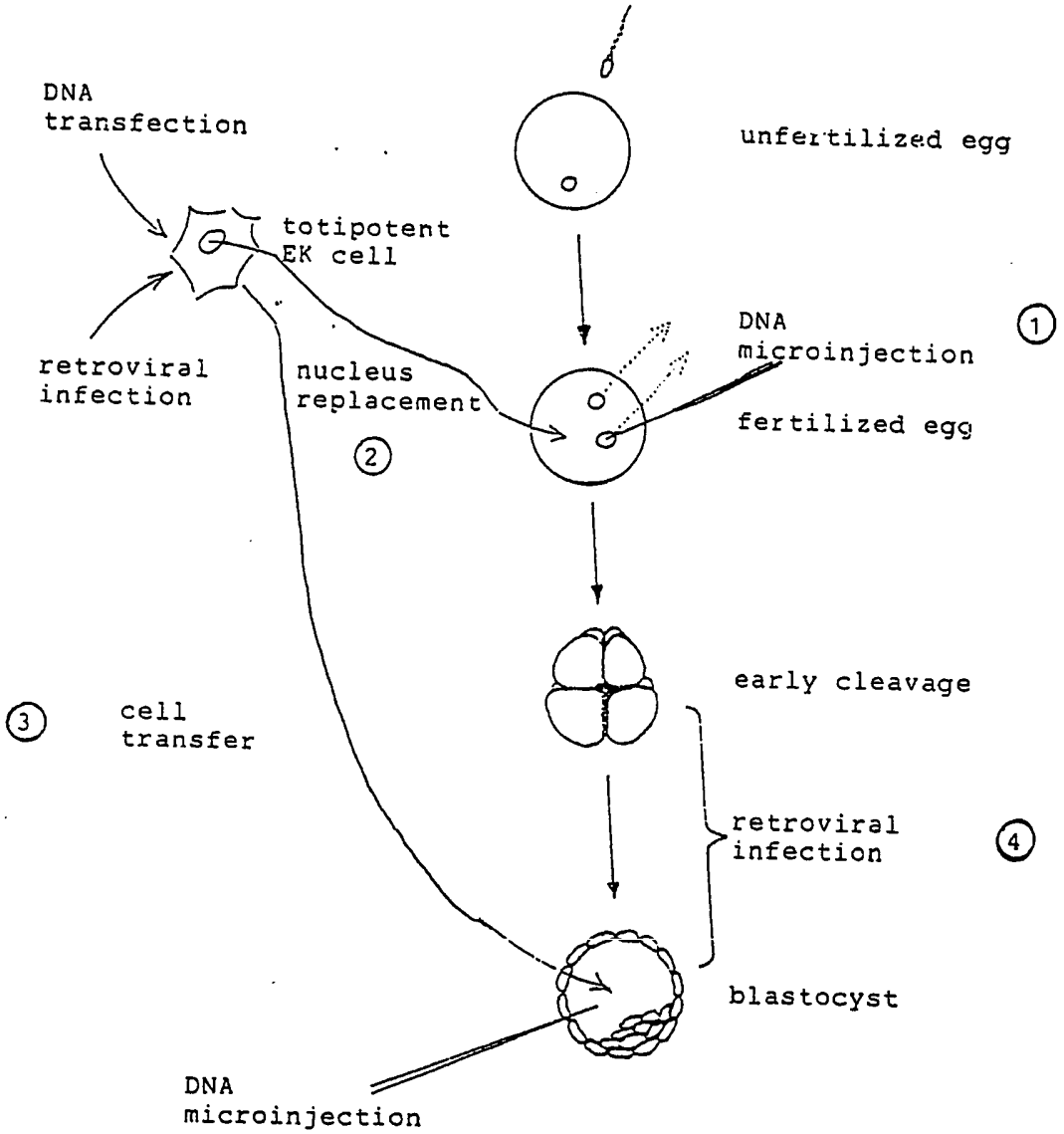


Figure 1

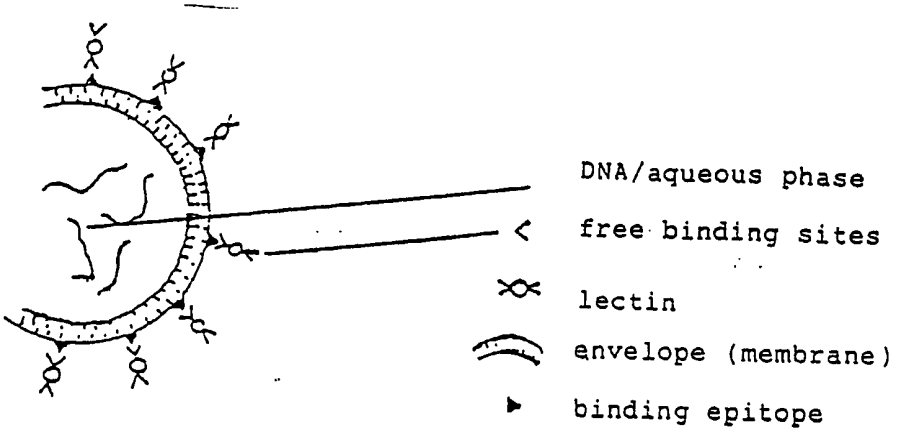
DNA/aqueous phase
free binding sites
lectin
envelope (membrane)
binding epitope

Figure 2

# INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 87/00123

## I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [8]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00; A 61 K 35/52; A 01 K 1/00

## II. FIELDS SEARCHED

| Minimum Documentation Searched [7] | |
|---|---|
| Classification System | Classification Symbols |
| IPC[4] | C 12 N; A 61 K |

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

## III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

| Category [10] | Citation of Document, [11] with indication, where appropriate, of the relevant passages [12] | Relevant to Claim No. [13] |
|---|---|---|
| A | WO, A, 82/04443 (OHIO UNIVERSITY) 23 December 1982 see page 8, lines 23-35; page 9, lines 1-3 -- | 1-19 |
| A | GB, A, 2144542 (N.L. FIRST et al.) 6 March 1985 see example VII -- | 1-19 |
| A | WO, A, 85/01856 (J.M.J. DE WET) 9 May 1985 see pages 3-5 | 1-19 |

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* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

## IV. CERTIFICATION

| Date of the Actual Completion of the International Search | Date of Mailing of this International Search Report |
|---|---|
| 22nd June 1987 | 27 JUL 1987 |
| **International Searching Authority** | **Signature of Authorized Officer** |
| EUROPEAN PATENT OFFICE | M. VAN MOL |

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/EP 87/00123 (SA 16645)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 03/07/87

The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|---|---|---|---|---|
| WO-A- 8204443 | 23/12/82 | EP-A- | 0081570 | 22/06/83 |
| GB-A- 2144542 | 06/03/85 | FR-A- | 2550218 | 08/02/85 |
| | | DE-A- | 3427553 | 14/02/85 |
| | | JP-A- | 60044869 | 11/03/85 |
| | | AU-A- | 3115984 | 07/02/85 |
| | | NL-A- | 8401137 | 01/03/85 |
| WO-A- 8501856 | 09/05/85 | EP-A- | 0160692 | 13/11/85 |